The Root-Knot Nematode Producing Galls on *Spartina alterniflora* Belongs to the Genus *Meloidogyne*: Rejection of *Hypsoperine* and *Spartonema* spp.

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Abstract: Root-knot nematodes are a major group of plant-parasitic nematodes, but their sister group within the Tylenchida remains to be identified. To find the sister group and for any investigation of the evolutionary biology of the genus *Meloidogyne*, it would be useful to identify the most basal species within Meloidogyninae. *Meloidogyne spartinae*, a root-knot nematode parasitic on cordgrass (*Spartina* spp.), constitutes a potentially interesting early diverging (or at least highly divergent) root-knot nematode because it was originally described in a different genus, *Hypsoperine* (and later *Spartonema*), due to its unique anatomy and biology (although it was later put in synonymy by some, but not all, taxonomists). We have sequenced the whole 18S rDNA of this species and compared it to other sequences of this region that are available in GenBank for numerous *Meloidogyne* species. Phylogenetic analysis unambiguously locates the branch corresponding to *M. spartinae* as a lately diverging species, more closely related to *M. maritima*, *M. duytsi* or the *M. ardenensis-hapla* group. Thus, the distinction of a separate genus (*Hypsoperine* or *Spartonema*) for this species is not justified.

Key words: Molecular biology, taxonomy, phylogeny, Tylenchida, Meloidogyninae, *M. spartinae*, 18S ribosomal DNA

Because they constitute major pests among phytoparasitic nematodes, the root-knot nematodes (*Meloidogyne* spp.) have received growing interest among plant nematologists. Two species of this genus, *M. incognita* (Kofoid and White, 1919) and *M. hapla* Chitwood, 1949, will soon be the first plant-parasitic nematode species to have their genomes sequenced (Bird, 2005; Castagnone-Sereno, 2006). Their homogenous morphology, wide host range (with numerous species being polyphagous) and reproductive biology, often characterized by parthenogenesis (eliminating the possibility of conducting experimental crosses to investigate species limits and relationships), have motivated the use of molecular tools to identify species within this speciose genus (at least 79 species already described [Siddiqi, 2000]), and to establish their phylogenetic relationships (Castagnone-Sereno et al., 1993; Hugall et al., 1994, 1999; Chen et al., 2003, Skantar and Carta, 2004; Scholl and Bird, 2005). Recent investigations of the molecular phylogeny of Tylenchida based on 18S sequences have deeply modified the conception of the phylogeny and taxonomy of Meloidogyninae. In particular, several studies suggest that the family Heteroderidae, which was considered until now as being formed by two sister sub-families (Heteroderinae and Meloidogyninae), is a polyphyletic group (Baldwin et al., 2004; Holterman et al., 2006, Subbotin et al., 2006). While the Heteroderinae are nested within the Hoplolaimidae, Meloidogyninae correspond to a distantly related group nested within the Pratylenchidae. However, the sister group of Meloidogyninae remains to be identified (Subbotin et al., 2006). To locate the sister group, and for any investigation of the evolutionary biology of the genus *Meloidogyne*, it would be useful to identify the most basal species within Meloidogyninae. However, to date, *Hypsoperine* is the only genus other than *Meloidogyne* that has been recognized within the Meloidogyninae. This genus was first distinguished from *Meloidogyne* spp. by Sledge and Golden (1964) for *Hypsoperine graminis* Sledge and Golden, 1964, a North American species parasitic on St. Augustine grass, *Stenotaphrum secundatum* (Walt.) Kuntze, and for *H. acronea* Coetzee, 1956 (Coetzee, 1956), an African species found on *Sorghum bicolor* (L.) Moench spp. *bicolor*. The authors "believe that this form represents a new genus which occupies a position between *Heterodera* and *Meloidogyne*, being closer to the latter;" they highlight that "the posterior portion of the female body is drawn out into a distinct rounded protuberance on which are situated vulva and anus" and the presence of a "thick cuticle." In 1965, Rau and Fassuliotis described *Hypsoperine spartinae* Rau and Fassuliotis, 1965, which is associated with *Spartina alterniflora* Loisel., although they consider that "some of the characters of their new species do not correspond with the *Hypsoperine* as outlined by Sledge and Golden." This species presents the largest dimensions of the second-stage juvenile within the Meloidogyninae, a very thin female cuticle, no gelatinous matrix (the eggs are deposited free in the gall) (Eisenback and Hirschmann, 2001), the smallest haploid chromosome number of the subfamily (Triantaphyllou, 1987), and an obligatory amphimictic mode of reproduction (Triantaphyllou, 1990). In 1969, a last species, *H. ottersoni* Thorne, 1969, which infests reed canarygrass, *Phalaris arundinacea* L., was added to the genus *Hypsoperine* (Thorne, 1969).

In 1968, Whitehead synonymized *Hypsoperine* with *Meloidogyne*, and this opinion was also shared by Jepson

(1987), Luc et al. (1988) and Eisenback and Triantaphyllou (1991). However, there was no consensus on the taxonomic status of the *Hypsoperine* genus (see for example Allen and Sher, 1967, or Triantaphyllou, 1990, who reported that "[its] generic status has been a controversial subject among nematode taxonomists"). This genus was considered valid by Golden in 1971 and by Handoo et al. in 1993, while Siddiqi (1986) split the genus in two, with *H. acronea, H.graminis* Sledge and Golden, 1964, *H. megriensis* Poghosyan, 1971, *H. mersa* Siddiqi and Booth, 1991, *H. ottersoni* and *H. propora* Spaull, 1977, staying in the genus *Hypsoperine,* while *H. spartinae* was considered a separate genus named *Spartonema.* In 2000, Siddiqi synonymized *Hypsoperine* with *Meloidogyne* but still considered that *Spartonema spartinae* (to which was added *S. kikuyensis*) should be kept in a distinct genus (Siddiqi, 2000).

Aside from the purely taxonomical issue of the genus name of the root-knot nematode producing galls on *Spartina alterniflora* and evaluating the validity of the genus *Hypsoperine* or *Spartonema,* we were mainly concerned with the phylogenetic position of this species relative to the other Meloidogyninae species. In particular, several characteristics concerning its morphology, its cytology or its reproductive mode suggest that it could be a rather divergent species of *Meloidogyne* and, hence, could be an interesting taxon to: (i) constitute an outgroup for a phylogeny of the *Meloidogyne* species, (ii) avoid long-branch attraction in broader phylogenetic investigations including Meloidogyninae and other Tylenchida families or sub-families and (iii) identify the sister group of the Meloidogyninae. For those reasons, we sequenced the 18S gene of the root-knot nematode associated with *Spartina* (to our knowledge, the first sequence for this species available in GenBank), added this sequence to a large data set of other Meloidogyninae species already available in GenBank and conducted phylogenetic analyses to identify the location of this species within the phylogenic tree of the Meloidogyninae.

Materials and Methods

Roots of *S. alterniflora* were collected 19 June 2005, from Cattus Island County Park near Toms River in Ocean County, NJ. Galls on the roots were dissected, and the nematodes (females, males and juveniles) extracted were identified morphologically using the keys and diagnostic characters provided by Rau and Fassuliotis (1965), Whitehead (1968) and Eisenback and Hirschmann (2001). In particular, the identification of the host plant (on which no other root-knot nematodes have been reported to date), the size of the juveniles (>750 µm, the longest among root-knot nematodes) and their characteristic long bulbous tail easily allowed the identification of the species (photographs available upon request).

Two primers (SSU-tyl-F GAAACTGCGTACGGCT-CATT and SSU-tyl-R GGTTCAAGCCACTGCGATTA) allowing amplification of the entire 18S gene (1,664 bp) were designed from an alignment of 18S complete sequences of 62 Tylenchida species available in GenBank. DNA was extracted as described by Plantard and Porte (2003) except that one female was used instead of one juvenile. PCR reactions were done in 10 µl final volume containing 1 µl of DNA extraction, 1X reaction buffer (containing 10 mM tris-HCl, pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 0.8 mM dNTP, 1µM of each primer, 0.5 unit Taq polymerase (AmpliTaq, Applied Biosystems). Amplifications were performed with a PTC-100 (MJ Research, Inc.) thermocycler using the following program: (i) one denaturation cycle at 94°C for 1 min, (ii) 30 cycles with 50 sec at 94°C, 50 sec at 56°C and 1 min 50 sec at 72°C, (iii) final step at 72°C for 5 min. PCR amplifications were checked with a 1% agarose gel electrophoresis. The amplified DNA was excised from the gel using MinElute Gel extraction Kit (Qiagen) and cloned into *E. coli* TOP10 strain with TA Cloning kit and pCR2.1 plasmid (Invitrogen). The transformants were screened by PCR with M13 universal primers (M13 forward-GTAAAACGACGGCCAGT, M13 reverse-AACAGCTATGACCATG) in 30 µl final volume containing the selected clone in 10 µl of sterilized distilled water, 1X reaction buffer (pH 8.5), 1.5 mM $MgCl_2$, 0.8 mM dNTP, 0.46 µM of each primer, 0.025 unit Taq polymerase (GoTaq Flexi, Promega). Amplifications were performed with a PTC-100 (MJ Research, Inc.) thermocycler and the following program was used: (i) one denaturation cycle at 96°C for 5 min, (ii) 30 cycles with 1min at 96°C, 1min at 55°C and 3 min at 72°C, (iii) final step at 72°C for 5 min. PCR amplifications were checked with a 0.8% agarose gel electrophoresis. The amplicons were cleaned using a sephadex G50 matrix (Amersham Biosciences) before completing the sequencing reaction (Macrogen, Inc.). Both strands were sequenced by the use of seven sequencing primers, including several internal primers, and this sequence is available in GenBank under the accession number EF189177.

As many 18S sequences of *Meloidogyne* spp. as possible were retrieved from GenBank using BLASTN and our sequence as a request (Table 1). Only the longest sequences (i.e., more than 1,500 nucleotides) were kept. Based on the recent finding concerning the location of the Meloidogyninae within the Pratylenchidae (Holterman et al., 2006; Subbotin et al., 2006), four outgroups from the Pratylenchidae family were used (two sequences from the genera *Hirschmanniella* and *Pratylenchus*). The total data set contained 56 sequences, to which was added our new sequence. Sequences that did not exhibit any polymorphism with another sequence within the data set were discarded (seven sequences). Alignments were conducted with Clustalx with default

TABLE 1. List of the species from which the 18S nucleotide sequence has been used in this study.

Species name	Accession number	Source
M. arabicida	AY942625	Tigano et al., 2006
M. ardenensis	AY593894	Holterman et al., 2006
M. arenaria	U42342	Georgi and Abbott, 1998
M. arenaria	AY268118	Lee and Williamson, 2003
M. arenaria	AF535867	De Ley et al., 2002
M. artiellia	AF248477	De Giorgi et al., 2002
M. artiellia	AF442192	De Ley et al., 2002
M. chitwoodi	AY593884	Holterman et al., 2006
M. chitwoodi	AF442195	De Ley et al., 2002
M. chitwoodi	AY593886	Holterman et al., 2006
M. chitwoodi	AY593887	Holterman et al., 2006
M. chitwoodi	AY593889	Holterman et al., 2006
M. chitwoodi	AY593888	Holterman et al., 2006
M. chitwoodi	AY593885	Holterman et al., 2006
M. chitwoodi	AY593883	Holterman et al., 2006
M. duytsi	AF442197	De Ley et al., 2002
M. ethiopica	AY942630	Tigano et al., 2006
M. exigua	AY942627	Tigano et al., 2006
M. exigua	AF442200	De Ley et al., 2002
M. fallax	AY593895	Holterman et al., 2006
M. hapla	AY593898	Holterman et al., 2006
M. hapla	AY593893	Holterman et al., 2006
M. hapla	AY593892	Holterman et al., 2006
M. hapla	AY268119	Lee and Williamson, 2003
M. hapla	AY942628	Tigano et al., 2006
M. ichinohei	AF442191	De Ley et al., 2002
M. incognita	U81578	Frisse et al., 1996
M. incognita	AY268120	Lee and Williamson, 2003
M. incognita	AY284621	Holterman et al., 2006
M. incognita	AY942624	Tigano et al., 2006
M. incognita	AF535868	De Ley et al., 2002
M. javanica	AY942626	Tigano et al., 2006
M. javanica	AY268121	Lee and Williamson, 2003
M. javanica	AF442193	De Ley et al., 2002
M. maritima	AF442199	De Ley et al., 2002
M. mayaguensis	AY942629	Tigano et al., 2006
M. microtyla	AF442198	De Ley et al., 2002
M. minor	AY593899	Holterman et al., 2006
M. naasi	AY593901	Holterman et al., 2006
M. naasi	AY593900	Holterman et al., 2006
M. naasi	AY593902	Holterman et al., 2006
M. oryzae	AY942631	Tigano et al., 2006
M. paranaensis	AY942622	Tigano et al., 2006
M. sp. 40	AY942634	Tigano et al., 2006
M. sp. 40	AY942635	Tigano et al., 2006
Meloidogyne spartinae	**EF189177**	**This study**
Hirschmanniella sp 1	AY284614	Holterman et al., 2006
Hirschmanniella sp 3	AY284616	Holterman et al., 2006
Pratylenchus crenatus	AY284610	Holterman et al., 2006
Pratylenchus thornei	AY284612	Holterman et al., 2006

options. Phylogenetic reconstructions using distance-based methods (Neighbor Joining using Kimura-2-parameters, Juke & Cantor, Tamura-Nei or logdet distances) were conducted with MEGA3 software (Kumar et al., 2001). For phylogenetic analyses based on Maximum Likelihood (ML), the appropriate model was selected using Modeltest (Posada and Crandall, 1998) and the likelihood settings of the selected model were used in PAUP*4b.10 (Swofford, 2002). To evaluate alternative topologies where *M. spartinae* was constrained

to belong to different groups, Kishino-Hasegawa (KH) tests were conducted using PAUP*4b.10 (two-tailed KH test with 1,000 bootstrap replicates and an estimated RELL distribution).

RESULTS

According to both hierarchical likelihood ratio tests and the Akaike information criterion, the selected ML model using Modeltest was the GTR+I+G model (General Time Reversible model, with a proportion of invariable sites and among-site rate variation defined by a gamma distribution). Among the outgroups, while a sister group relationship between the two *Hirshmaniella* species was always observed (BV 99 or 100%), the two *Pratylenchus* species were much more distantly related, with a sister group relationship between those two congeneric species in half of the analyses but never with a high BV (35–52%; Fig. 1). The *Meloidogyne* genus always formed a monophyletic group (BV 100% in the ML tree), except for the most basal and divergent taxa, *M. ichinohei* Araki, 1992, which clustered as the sister group of the Pratylenchid outgroups in some NJ trees. The remaining *Meloidogyne* species, including the root-knot species associated with *Spartina*, always formed a monophyletic group with BV at 96% (ML) or 99 or 100% (NJ).

Within those *Meloidogyne* species, a first clade corresponded to *M. artiellia* Franklin, 1961, whereas all the other species formed a separate clade with BV at 99 or 100%. Within this second clade (which includes all the species except *M. ichinohei* and *M. artiellia*), three to six groups could be recognized, depending on which data set (pairwise or complete deletion, default or manually corrected alignment) or distance was used. A relatively well supported group (BV from 69% in the ML tree to 77–95% in the NJ trees) was formed by *M. ardenensis* Santos, 1968, *M. microtyla* Mulvey et al., 1975, and *M. hapla* (hereafter called the *M. ardenensis-hapla* group). *Meloidogyne spartinae* was carried by a relatively long branch; this is due to a high number of singletons (25) found in this sequence relative to all other *Meloidogyne* species. However, careful examination of the sequence at each singleton position confirmed that those differences were not base-calling errors. In most cases, *M. spartinae* and *M. maritima* Jepson, 1987, were sister groups (and in particular in all the analyses based on pairwise deletion; BV ranging from 62–73% in the NJ trees and 88% in the ML tree), and this clade is the sister group of the *M. ardenensis-hapla* group. *Meloidogyne duytsi* Karssen et al., 1998, is more closely related to *M. maritima*, *M. spartinae* or the *M. hapla-ardenensis* group and thus appears either as the sister group of those species (BV 79% in ML tree), or as a sister group of the remaining species. All the remaining *Meloidogyne* species formed two last groups of species. A first group contained two well supported clades; the first clade con-
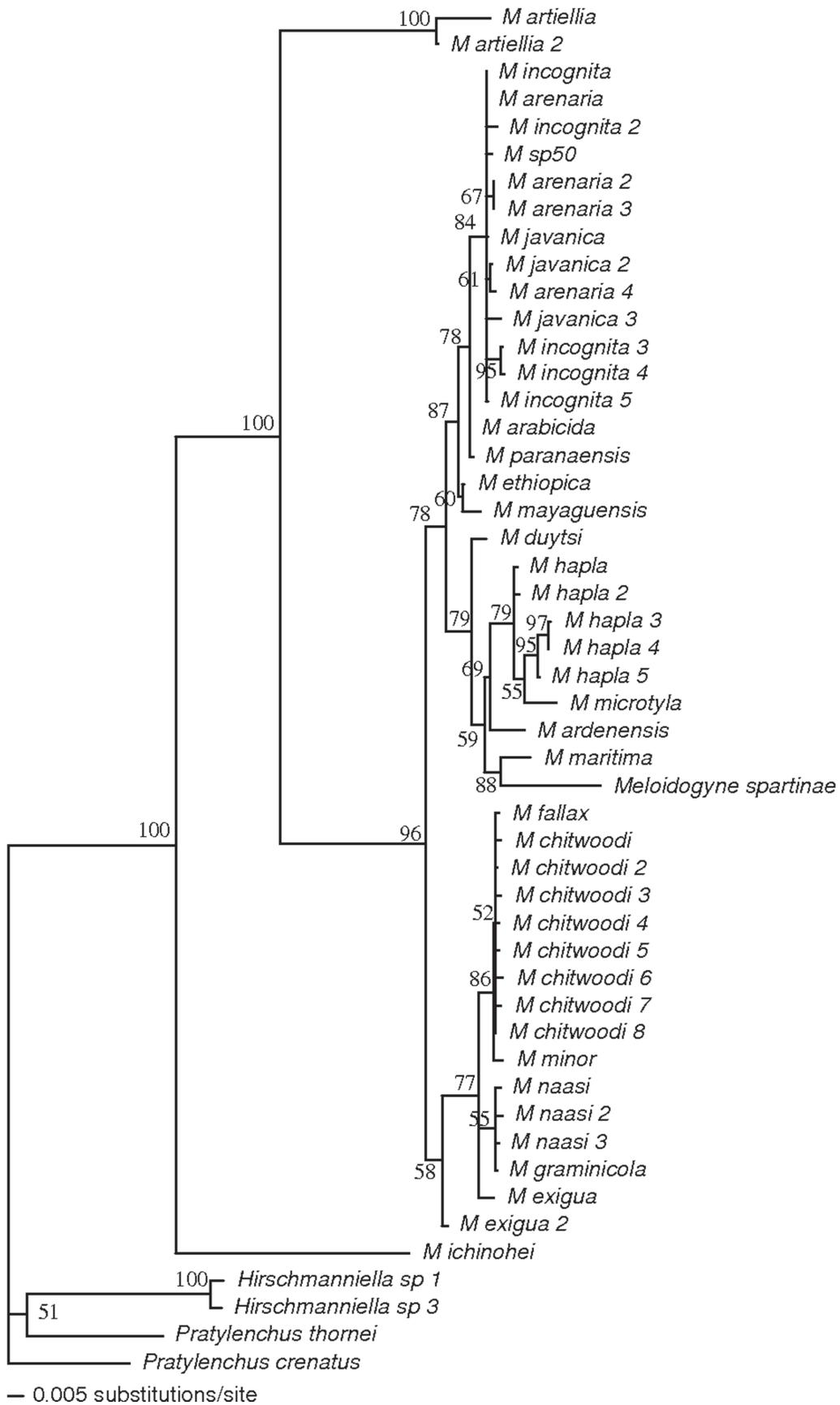
FIG. 1. Phylogeny of the *Meloidogyne* genus based on the 18S sequence. Maximum Likelihood tree obtained with PAUP*4b10, based on the GTR+G+ I model and likelihood settings determined by Modeltest. Only bootstrap values (based on 1,000 replications) superior to 50 are shown.

tained *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. incognita, M. javanica* (Treub, 1885) Chitwood, 1949, *M. mayaguensis* Rammah & Hirschmann, 1988, *M. paranaensis* Caeirno et al., 1996, *M. ethiopica* Whitehead, 1968, and *M. arabicida* Lopez and Salizar, 1989 (BV ranging from 87% in the ML tree to 94–100% in the NJ trees), and the second clade corresponded to *M. exigua* Göldi, 1892, *M. naasi* Franklin, 1965, *M. graminicola, M. chitwoodi* Golden et al., 1980, *M. fallax* Karssen, 1996, and *M. minor* Karssen et al., 2004 (BV ranging from 58% in the ML tree to 83–95% in the NJ trees or 77% in the ML tree to 99–100% in the NJ trees when excluding one *M. exigua* sequence). When 10 alternative topologies (placing *M. spartinae* with *M. artiellia*, or with *M. ichinohei*, or with the outgroups, etc.) were tested using the KH test, the highest likelihood score was obtained for the topology corresponding to *M. spartinae* clustering with the other *Meloidogyne* species, *M. artiellia* being the sister-group of this first cluster, then *M. ichinohei* being the sister-group of this second cluster (–ln L = 10,786.62; all other topologies tested with a significantly lower likelihood score, KH–test: $P < 0.05$).

DISCUSSION

While the phylogenetic relationships among all the *Meloidogyne* species are not fully resolved (in particular, the relative position of the three to six groups described above is not resolved; but see a more comprehensive study based on multiple gene analysis of EST data to confidently locate the position of the groups containing *M. chitwoodi, M. hapla, M. incognita* and *M. javanica*, [Scholl and Bird, 2005]), our analysis unambiguously places *M. spartinae* within the genus *Meloidogyne*. The exact location of *M. spartinae* is not fully resolved, but it is certainly not close to the early diverging species *M. ichinohei* or *M. artiellia*. The basal position of those two species (to which should probably be added the recently described *M. baetica* but for which an 18S sequence is not available to date [Castillo et al., 2003; Subbotin et al., 2006]) has already been stated by several authors (De Giorgi et al., 2002; De Ley et al., 2002; Tigano et al., 2005). Among the more lately diverging *Meloidogyne* species, *M. spartinae* is neither related to the *exigua-chitwoodi-fallax* group nor to the *arenaria-javanica-incognita* group. *Meloidogyne spartinae* is more closely related to the *M. ardenensis-hapla* group, *M. maritima* or *M. duytsi*. Concerning the ecology of *M. spartinae,* it is interesting to note that those last two species are also associated with Poaceae species restricted to the seashore, namely *Ammophila arenaria* (L.) Link and *Thinopyrum junceiforme* (A. & D. Löve) A. Löve (previously known as *Elymus farctus*) in Europe (with a vicariant species, *M. sasseri,* associated with *A. breviligulata* Fern. on the east coast of the US, but for which no 18S sequence is available [Handoo et al., 1993]), like *Spartina*

spp., although cordgrass has a different ecology because it is found in the intertidal zone and not on sand dunes.

Meloidogyne graminis is another *Meloidogyne* species once included in the *Hypsoperine* genus (cf. Sledge and Golden, 1964). Because a short 18S sequence (637 bp) of *Meloidogyne graminis* is available in GenBank (Powers et al., 2005), this species could also be compared to *M. spartinae*. Phylogenetic analysis (data not shown) provides a topology similar to that previously described, with *M. graminis* being the sister group of *M. spartinae* (although with a weak bootstrap value of 47%, probably due to the shorter sequence), close to *M. maritima* or the *M. hapla-ardenensis* group. Thus, the close relationship between *M. graminis* and *M. spartinae* was correctly identified by the authors that described those species, but their supposedly distant relationship with other root-knot nematodes leading to the description of a separate genus (*Hypsoperine* by Sledge and Golden, 1964, or *Spartonema* by Siddiqi, 1986) was erroneous.

Thus, our analysis, which is based on molecular investigations, confirms the statements made by Triantaphyllou (1987) based on anatomical observations of the structure of the reproductive system and by Eisenback and Hirschmann (2001) based on morphological investigations that *M. spartinae* is a member of the genus *Meloidogyne*. Our results confirm the synonymyzation of *Spartonema* with *Meloidogyne* as previously proposed by Jepson (1987). Contrary to the hypotheses made by several authors (Triantaphyllou, 1987, 1990; Eisenback and Hirschmann, 2001) that the atypical chromosome number of this *Meloidogyne* species (a unique feature shared only with *M. kikuyensis*) represents the ancestral form, our results suggest instead that it is a derived character state arising from the 13 to 19 chromosomes of most other *Meloidogyne* species. Finally, because of the internal position of *M. spartinae* within the *Meloidogyne* phylogenetic tree, our results highlight; (i) the importance of the highly divergent species within the *Meloidogyne* genus (which are *M. artiellia, M. ichinohei* and potentially *M. baetica*) for investigations on the evolutionary biology of root-knot nematodes, and (ii) the necessity of getting additional sequences of other poorly known *Meloidogyne* species (including species like *M. spartinae* that are not associated with crops) such as *M. kikuyensis.*

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